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Rapid HPLC method for the determination of vitamin A and E and cotinine concentration in human serum in women with CIN and cervical cancer

Szybka metoda oceny stężenia witaminy A, witaminy E oraz kotyniny w surowicy krwi kobiet z śródnabłonkową neoplazją (CIN)i rakiem szyjki macicy

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Abstract

Objective: *The aim of this study was to elaborate on the analytical method for quantitative determination of retinol and α-tocopherol in serum of women diagnosed with CIN and cervical cancer. The basic problem in the analysis of the vitamins content in biological material is their low physiological concentration level and instability. Liquid chromatography with diode array detector (DAD) was applied.*

Material and methods: *The material consisted of serum and urine collected from 12 women diagnosed with cervical intraepithelial neoplasia (CIN) and 16 diagnosed with cervical cancer.*

The method was evaluated for the following parameters: linearity, recovery, sensitivity, precision, accuracy, selectivity, stability, limit of quantification (LOQ) and limit of detection (LOD).

Results: *Results showed good linearity ($r^2 \geq 0{,}99$) in the range 0,1µg/ml-10mg/ml for retinol and 0,25µg/ml-15µg/ml for α-tocopherol. The Lower Limit of Detection was 0,15µg/ml for vitamin E and 0,05µg/ml for vitamin A. The within-run R.S.Ds were below 5,2% at all concentration levels and the between-run R.S.Ds were below 10,0% at all concentration levels.*

Conclusions: *The advantage of this method is that it measures both compounds in a more rapid, reproducible and accurate manner when compared to the previous HPLC studies. The compounds (vitamin A and E and internal standards) are measured in the same sample at the same time.*

Quantitative determination of cotinine may reveal active smokers and subjects exposed to environmental tobacco smoke, which is independent measurable carcinogenetic co-factor.

The following study is a part of a project determining non-viral causative agents in cervical carcinogenesis.

Key words: **vitamin A** / **vitamin E** / **retinol** / **alpha-Tocopherol** / / **high pressure liquid chromatography – HPLC** / **cotinine** /

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Streszczenie

Cel pracy: *Celem niniejszej pracy jest ocena przydatności analitycznej metody oznaczania ilościowego retinolu i α-tokoferolu w surowicy kobiet z rozpoznaną śródnabłonkową neoplazją (CIN) i rakiem szyjki macicy. Głównym problemem w analizowaniu zawartości witamin w materiale biologicznym jest ich niskie fizjologiczne stężenie i niestabilność. W niniejszym badaniu zastosowano metodę ciekłej chromatografii z detektorem diodowym (DAD).*

Materiał i metodyka: *Materiał badawczy stanowiło osocze i mocz, 12 kobiet z rozpoznaną śródnabłonkową neoplazją szyjki macicy (CIN) i 16 z rakiem szyjki macicy.*

Metodę oceniono pod kątem następujących parametrów: liniowość, powtarzalność, czułość, precyzja, dokładność, selektywność, stabilność, granica kwantyfikacji (limit of quantification - LOQ) i detekcji (limit of detection - LOD).

Wyniki: *Uzyskano wysoką liniowość (r^2 ≥0,99) w zasięgu 0,1μg/ml-10mg/ml dla retinolu i 0,25μg/ml-15μg/ml dla α-tokoferolu. Dolna granica wykrywalności wyniosła 0,15μg/ml dla witaminy E i 0,05μg/ml dla witaminy A. Zasięg metody R.S.Ds wyniósł 5,2% we wszystkich poziomach stężeń a interwałowy zasięg R.S.Ds poniżej 10,0% we wszystkich poziomach stężeń.*

Wnioski: *Zaletą zbadanej metody jest fakt, iż można przy jej pomocy uzyskać wyniki w szybszy, bardziej powtarzalny i dokładniejszy sposób, niż w opisywanych w literaturze badaniach z użyciem HPLC. Dodatkową zaletą stosowanej metody jest możliwość pomiaru z tej samej próbki witamin A i E.*

Ilościowa ocena stężenia kotyniny może wskazać pacjentki, będące aktywnymi palaczkami lub narażonymi na palenie bierne. W aspekcie karcinogenezy w obrębie szyjki macicy może być to cenna wiadomość dotycząca istotnego a często zatajanego kofaktora nowotworzenia

Niniejsze badanie jest częścią projektu oceniającego pozawirusowe czynniki onkogenne w procesie karcinogenezy w obrębie szyjki macicy.

Słowa kluczowe: **witamina A** / **witamina E** / **retinol** / **alfa-tokoferol** / / **wysokosprawna chromatografia cieczowa – HPLC** / **kotynina** /

Introduction

Vitamins A and E, their functions and deficiency syndromes

Vitamins are small chemical molecules, essential for optimal health. Their biological role, as coenzymes, is attributed to the control of catalytical reactions. They have influence on numerous hormone-dependent processes.

In small amounts, vitamins are essential for health. However, neither animals nor humans are able to produce enough of them and therefore vitamins must be supplemented. Thus, the main source of vitamins or their precursors is a balanced diet. It should be emphasized that a balanced diet consists of sufficient amounts of vitamins.

Deficiency of vitamins may be dietary in orgin, or may occur due to disturbances in intestinal absorption, presence of antivitamins or liver damage. In case of certain vitamins deficiency is caused by their impaired synthesis by intestinal flora or prolonged treatment with antibiotics. Due to their solubility, vitamins are divided into fat- (A, D, E, K), and water-soluble (group B, C, PP) which act as coenzymes. [1]

Monitoring of the status of vitamins A and E is important to detect inadequate dietary intake of these vitamins or a possible deficiency of either retinol-binding protein, protecting retinol from degradation pathways or of α-tocopherol transfer protein (α-TTP), which incorporates α-tocopherol into very-low-density lipoproteins in the liver. [2, 3, 4, 5, 6, 7, 8, 9, 10].

In this paper we discuss a procedure for simultaneous detection of vitamins A and E in serum using HPLC with DAD method.

Vitamin A

Vitamin A, the most important form of which is retinol, is produced in the intestinal mucosa from provitamin A (known as beta-carotene) through an oxidative cleavage reaction.

Retinol is oxidized in vivo to aldehyde retinal (the form used in visual pigment) and retinoid acid. Both of them are known as retinoids and are active forms of vitamin A. Vitamin A, produced and absorbed in the proximal part of the intestine, is transported in chylomicrons via enterohepatic circulation to the liver for esterification and storage. When retinol esters in the liver are mobilized, released retinol is then bound to α-1-globulin and albumin. Most of the vitamin A reserves in a body are stored in the liver. Important dietary sources of vitamin A are animal-derived (fish liver, cod-liver oil, milk, eggs, butter).

It should be underlined that human breast milk contains twice as much vitamin A as whole milk of a cow. In humans, beta-carotene remains the basic source of vitamin A. Yellow and leafy green vegetables such as carrots provide large amounts of carotenoids, many of which are provitamins that can be metabolized in the active form in vivo. Thus, the plants neither produce nor contain vitamin A, they merely supply provitamins for its synthesis. The rate of vitamin A production from α-carotene is influenced by the actual demands of the body. Therefore, its excessive dietary intake can lead to vitamin A toxicity.

Molecular mechanism of retinoids' influence is based on their binding to intracellular receptor, impact on gene expression and, consequently, on protein synthesis. This mode of action is responsible for synthesis of a dozen proteins, amongst them: EGF receptor, kreatine, cell-growth control polyamide.

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The most important function in visual process involves predominantly one form of vitamin A, namely retinal. Retinal enters into the composition of rhodopsin, the most light-sensitive pigment and thus highly important in perception of reduced light. Rhodopsin, under the influence of light, undergoes a sequence of changes to ultimately disintegrate to trans-retinal and opsin. In the process, a nerve impulse is generated which assures good vision. Vitamin A deficiency leads to deprivation of rhodopsin, night blindness and xerophthalmia.
Vitamin A prevents the keratinization of mucous-secreting epithelium of gastrointestinal and respiratory tract. It accelerates cell division and growth of fibroblasts, thus acting as the growth factor, predominantly in young organisms. Vitamin A plays an important role in spermatogenesis and embryogenesis. There is evidence that vitamin A may inhibit the growth of experimental breast cancer.

Recommended daily intake of vitamin A is 500IU. The demand for vitamin A increases significantly during pregnancy and breast feeding.

Vitamin E

Vitamin E is widely distributed in nature, particularly large amounts may be found in vegetable oils (soy), vegetables, dairy products and nuts. A group of eight closely related compounds, known as tocopherols, exhibit vitamin E activity, but a tocopherol is the most active one. It constitutes (up to) 90% of all body tocopherols.

As it is currently known, tolopherols are exclusively produced by plants. The absorption of vitamin E, as of all fat-soluble vitamins, takes place in ileum, requires normal biliary tract and pancreatic function, and depends on diet. If the intestinal absorption is impaired, the intramuscular oil solutions of vitamin E may be administered. Vitamin E is excreted with bile, as glucuronide, and by the kidneys, as tocopheronic acid.

This essential nutrient belongs to the group of antioxidants whose function is scavenging free radicals formed in redox reactions throughout the body. It plays a role in termination of oxidative-generated lipid peroxidation chain reactions, particularly in cellular membranes that are rich in polyunsaturated lipids. Vitamin E is a component of LDLs and protects them from oxidative damage.

Hypovitaminosis E is associated with inadequate bile release and small intestinal disease. Vitamin E deficiency contributes to neurologic manifestations, peripheral and central neuropathies. In case of adults, unlike in children, neuropathies are not a feature of the deficiency state and may be present after many years of inadequate intake. Vitamin E deficiency may affect mature red cells by shortening their life span. Fertility disorders may be seen in women (impaired implantation), as well as men (reduced spermatogenesis). It has been suggested that vitamin E may have protective effects against atherosclerosis and cancer, the two most common causes of death in developed countries. It is prescribed in treatment of anemia, blood vessel spasm disorders, collagenoses and atherosclerosis. Vitamin E can be used in recurrent abortions, oligospermia and muscular atrophies. However, therapeutic effect of vitamin E has not yet been conclusively stated and defined.

Daily requirement of vitamin E is 10-3-mg.

Cotinine

Nicotine is a pyridine alkaloid that constitutes 85 to 90% of all alkaloids in tobacco leaves. Its main metabolite (80%) is cotinine; the metabolism is shown in Figure 1.

The sources of nicotine and cotinine in biological fluids are tobacco smoking and medicines containing nicotine (chewing-gum, plasters, inhalers) [11, 12].

Cotinine – the main nicotine metabolite – is eliminated from the body for a longer period of time than nicotine.

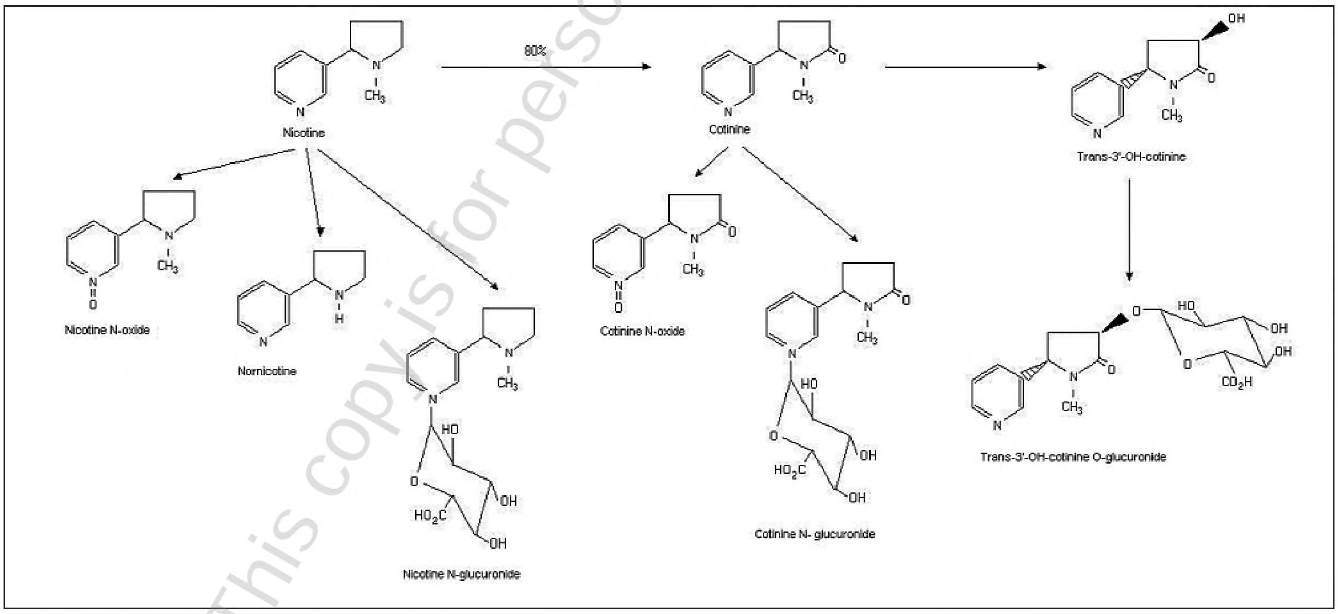

Figure 1. Nicotine metabolism [12,14].

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Its biological halftime is 17 hours, clearance 0,04 l/min, distribution volume 55 l (0,9 l/kg) [13]. The main way of cotinine and its metabolites elimination is via urine. Cotinine is one of the best and most useful biomarkers of tobacco smoke exposition [14, 15, 16].

The urine cotinine concentration reflects exposition to nicotine and tobacco smoke in the last 2-3 days. Research showed that urine cotinine concentrations may be useful in distinguishing active smokers from the ones exposed to environmental tobacco smoke [15]. The cotinine concentration in active smoker urine is higher than 100ng/ml and can be higher than 3000ng/ml. The cotinine concentration in the urine of a no-smoker exposed to environmental tobacco smoke one ranges from 25 to 100ng/ml [15, 16, 17].

Material and methods

28 patients, aged 19 to 67 years, diagnosed with pre-invasive or invasive cervical lesion were included in the study in accordance with ethical approval regulation by Local Research Ethic Committee of Jagiellonian University in Krakow. The basis for the diagnosis were: punch or cone biopsy specimens obtained from consecutive pre-invasive CIN1 (n=3), CIN2 (n=3), and CIN3 (n=3) lesions at the *Department of Gynecology and Obstetrics, Jagiellonian University Medical College, Krakow* between 2004 and 2006. Additionally, specimen of primary squamous cell carcinoma of the cervix CaCx (n=16) were included into the study from patients who had undergone the radical hysterectomy with pelvic lymph node dissection. The material for this pilot study included 20ml of blood and 100ml of morning urine.

Chemicals and reagents

Retinol, α-tocopherol, retinol acetate, α-tocopherol acetate and ascorbinic acid in substance were provided from Sigma-Aldrich. Methanol, acetonitryl, hexane, water and dichloromethane used in this determination were of analytical grade (Merck). Ethanol was from PoCH (Gliwice). Chemicals used for cotinine determinations were: methanol HPLC Grade (Merck), octanesulfonic acid sodium salt monohydrate (Fluka), dichloromethane HPLC Grade, acetonitrile HPLC Grade, phosphoric acid, K2HPO4, 2-propanol, cotinine standard (Sigma), NaOH, HCl (POCh), deionized water.

Chromatographic conditions

The chromatographic separations and quantitative determination were performed on a high performance liquid chromatograph Agilent 1100 series equipped with a pump, degasser, autosampler and DAD detector. A Supelco analytical column (Discovery C18 column, 5mm particle size, 250x4.6mm) as the stationary phase was used.

Methanol was used as the mobile phase. The flow rate was 1.0 ml/min. Detection was monitored at two different wavelengths: l=292nm for tocopherols and l=325nm for retinols determination.

Cotinine was determined by high performance liquid chromatography (HPLC) with UV detector (254nm).

The equipment included: HPLC Merck Hitachi Pump L6200A, Detector Spectra 100 Thermo Separation Products, 4880 integration system, centrifuge MPW 220, multi-block heather Lab-Line, ultrasonic cleaner Sonic-2, vacuum apparatus Alltech, vortex TH-3S TechnoCartel, pH meter MAT1202-SM.

The resolution was performed on Supelcosil LC-8 column (25cm, 4.6mm ID). The mobile phase was deionized water 88%, acetonitrile 12 %, sodium octasulphoniane 1.1g/l, dicalium phosphate 5.95 g/l; pH was adjusted to 4.7 with the use of phosphoric acid. The HPLC analysis was isocratic with constant flow rate 1ml/min. The Rheodyne injector loop was 100μl. The detector wavelength was 254nm.

Standards and samples preparation

The blood was extracted and left to spontaneous coagulation. Then the samples were centrifuged at 300 r.p.m. for 15 minutes, becoming excluded haemolysed samples. The clean serum was frozen and stored at the temperature of about -40°C. "Stock solutions" of retinol, retinol acetate, α-tocopherol and α-tocopherol acetate were prepared first in the mixture of hexane/dichloromethane (50/50 v/v) containing ascorbic acid (0,025%) to protect vitamins against oxidation.

After this, standard solution was diluted in a mixture of dichloromethane/methanol (50/50 v/v) and, finally, in methanol (100%) and stored at -22°C, without the access of light. The QC samples were prepared in human plasma and stored in the same conditions as the analytical samples. The calibration samples were prepared immediately before the analysis.

The preparation of samples was done following the procedure described by M.A. Rodriguez-Delgado at al. [13]. 100ml retinol acetate (internal standard I) and 100ml α-tocopherol acetate (internal standard II) and 200ml of ethanol were added at 200ml volume of serum.

The mixture was shaken for 5 minutes. Subsequently, 200ml water and 800ml of hexane was added to the mixture and after blending for 15 minutes it was centrifuged for 5 minutes at 4000 r.p.m., and next 600ml of the upper organic layer was extracted. The organic phase was evaporated to dryness and the residue was dissolved in 100ml of methanol/ethanol/hexane (88:10:2, v/v/v).

All samples were run in duplicate, one with and the other without internal standards.

At 20ml volume of this solution was injected directly into the batcher. The chromatographic separation took 18 minutes.

Cotinine extraction procedure

The liquid-liquid extraction was used for the preparation of the samples. 0.5ml of 1M NaOH and 50μl of norephedrine (internal standard) was added to 5 ml of urine and mixed. The samples were extracted with 5 ml mixture of dichloromethane: 2-propanol (9:1) (v/v) for 15 minutes. The organic phase was collected to cone tubes and evaporated in the air stream in 40°C. The residues after evaporation were dissolved in 400ml of mobile phase and injected to HPLC.

Calibration curves

For calibration curves the stock solutions were diluted with methanol to obtain a mixture of working standards. Water containing measured amounts of retinol and α-tocopherol was used for the analysis.

The samples of water were spiked with α-tocopherol to the following concentration: blank, 0.15; 0.25; 0.75; 1.5; 5.0; 10.0; 15,.0; 20.0mg/ml and with retinol to the following concentration: blank, 0.05; 0.10; 0.25; 0.5; 1.0; 2.5; 5.0; 10.0mg/ml. Retinol acetate and α-tocopherol acetate were used as the internal standards. The concentration of retinol was 10μg/ml and α-tocopherol acetate was 100μg/ml. The blank samples were water and serum. Calibration for cotinine was made using blank urine spiked with cotinine to obtain standards: 10, 50, 100, 200, 500, 1000ng/ml.

These calibration samples were analysed following the procedure described for sample preparation (2.3 section). The number of QC's per batch was six (three concentrations level in duplicate). They were assessed according to 4-6-20 rule.

Results

Specificity / selectivity

The specificity/selectivity of the method can be illustrated by comparing the chromatograms obtained after analysis of a test solution of the pure compound with the chromatograms of the independent blank serum. No interfering peaks can be seen if blank serum is used. The peaks of the retinol, retinol acetate (Figure 2), α-tocopherol and α-tocopherol acetate (Figure 3) were well-resolved and showed no interferences with endogenous or exogenous materials.

The retention time was: 4.54 minutes for retinol; 5.46 minutes for retinol acetate, 9.4 minutes for α-tocopherol and 11.35 minutes for α-tocopherol acetate.

Sensitivity

The lower detection limit depends on several parameters such as the mobile phase and the DAD monitor used. Under the experimental conditions described, the LOD (defined as three times the baseline noise) was approximately 0.15μg/ml for vitamin E and 0.05μg/ml for vitamin A. The lowest concentration of the calibration graph for vitamin E was 100ng/ml and for the vitamin A was 250ng/ml and it was the Lower Limit of Quantification.

For cotinine the limit of detection and the limit of quantification were 5ng/ml and 10ng/ml respectively.

Linearity

Detector response for vitamin A, E and internal standards was linear to at least 100mg/ml. The resulting data was plotted as peak height and peak area versus concentration and studied by the linear regression analysis. Tables 1 and 2 present the results for the assessment of the goodness of fit/lack of fit for both compounds (vitamin A and vitamin E) in serum. The goodness of fit was highly significant. A test for lack of fit indicated that the linear model is appropriate for establishing a relationship between the concentration and the response. In general, correlation coefficients above 0.98 were observed during the validation experiments.

The linearity for cotinine was 10-1000ng/ml. The urine samples with cotinine levels higher than 1000ng/ml were dissolved.

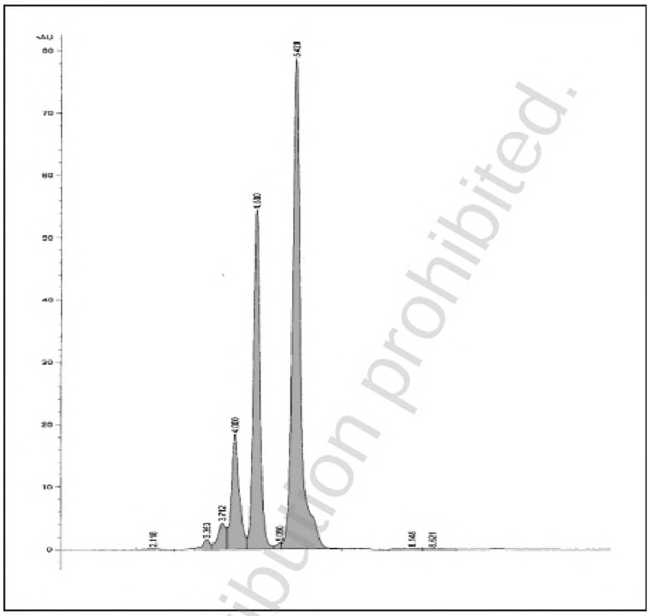

Figure 2. Chromatogram from DAD detector obtained at l=325nm. It shows the peaks of the retinol and retinol acetate.

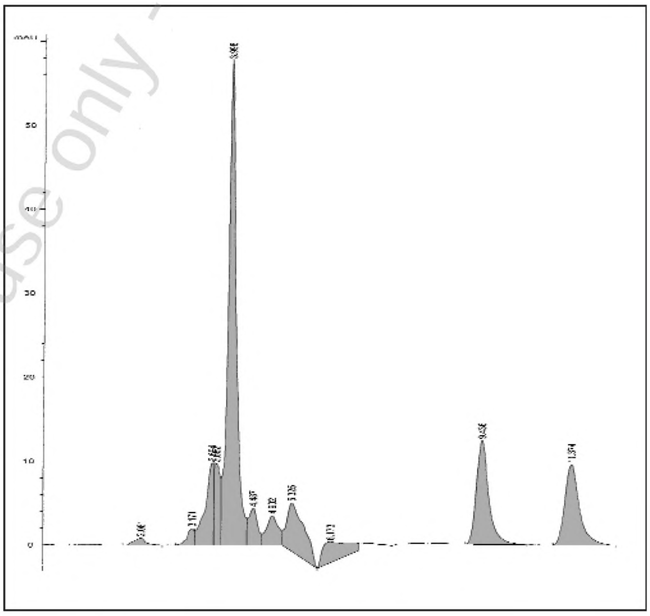

Figure 3. Chromatogram from DAD detector obtained at l=292nm. It shows the peaks of the α-tocopherol and α-tocopherol acetate.

Precision and accuracy

A summary of the results on precision and accuracy as derived from the measured concentration for the validation samples is presented in tables I and II.

The within-run R.S.Ds. were below 9.44% for tocopherol and below 9.52% for retinol at all concentration levels in serum. Criteria of acceptance are ±15% at all level concentrations only at the LLOQ are ±20% [13, 21, 22].

The coefficient of variance (CV) for series of cotinine 200ng/ml controls was 5.13%.

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Table I. Goodness of fit and lack of fit for Vitamin E (n=12).

Concentration (μg/ml)	mean	SD	R.S.D. (%)	Bias (%)
0.25	0.31	0.01	4.19	23.08
1.5	1.27	0.12	9.44	-15.01
10	9.55	0.89	9.37	-4.54

Table II. Goodness of fit and lack of fit for Vitamin A (n=12).

Concentration (μg/ml)	mean	SD	R.S.D. (%)	Bias (%)
0.25	0.26	0.02	9.52	4.82
1	1.06	0.05	5.18	6.15
5	5.22	0.06	1.06	4.47

Recovery

For determination of recovery of the analysis, the mean peak heights obtained for triplicate measurements were compared with the mean peak heights obtained from triplicate direct injections performed in the same run. The five recoveries thus obtained were used to calculate the mean recovery.

Discussion

Squamous cervical cancer arises from the metaplastic epithelium of the transformation zone (TZ) *(squamocolumnar junction)* and develop slowly through progressive dysplastic changes to carcinoma in situ and invasive cancer.

Cervical intraepithelial neoplasia (CIN) is divided into three stages according to the degree of epithelial dysplasia and differentiation.

Over the past few decades significant advances have been made in understanding the molecular genetics underlying the development of human cancers. However, we are still far from constructing complete sequelae of events leading to the development of invasive cervical cancer. There are many associated risk factors including number of sexual partners, parity, oral contraceptives use, smoking, immunological system alterations and lack of antioxidants [23] but persistent human papillomavirs (HPV) 16 and 18 infection is the most significant factor in aetiology of this disease [24].

The applied method to determinate retinol and a-tocopherol in serum uses an isocratic separation with only methanol as mobile phase at 1.0ml/min as flow rate which it allows a fast elution of both compounds from the column. This method do not require complex mobile phase. Applied DAD detector allows to measuring both vitamins in the same chromatographic separation. As can be observed the matrix effect obtained at 292nm can be eliminated measuring only α-tocopherol and α-tocopherol acetate (IS) at 292nm and retinol acetate (IS) and retinol at 325nm.

The advantage of this method is its high sensitivity and specificity. The preparation of samples was made following the proceeding described by M.A. Rodriguez-Delgado at al. [18] This technique of extraction is very simply and allowed analysis both compounds vitamin E and vitamin A in the same biological sample. Detection both vitamins have been developed and documented by analyses of serum obtained from gynaecology patients enrolled in a cervical carcinoma maintenance treatment.

The proposed method was applied to 12 women diagnosed with cervical intraepithelial neoplasia (CIN) and 16 with cervical cancer in order to determine serum concentrations of retinol and α-tocopherol in these patients. The mean age of patients was 39 (19-67).

The range of concentration of vitamin A in this group of 28 patients was 1.40mg/lserious one? -7.71mg/l and of vitamin E was 2.61mg/l-17.93mg/l. The minimal and maximal concentrations for both vitamins was measured for every subject. The mean concentration was 8.53mg/l for vitamin E and 3.75mg/l for vitamin A.

Cotinine in urine samples in the studied group ranged from 0.0ng/ml to 5917,4ng/ml. The concentration of cotinine in urine was in range 34.3-73.9ng/ml in 7 persons what indicates they were only exposed to environmental tobacco smoke. The concentration of cotinine in range 0.0-8.8ng/ml in 9 patients indicates a non-smoking person. The concentration of cotinine in range 274.0-5917.4ng/ml in 10 patients indicates active smokers.

The mean concentration of vitamin A and E were respectively 3.35mg/l and 8.29mg/l in the group of active smokers, 4.22mg/l and 8.65mg/l in the group of patients exposed to environmental tobacco smoke, 4.68mg/l and 10.32mg/l in the group of non-smokers.

Conclusions

Quantitative determination of cotinine may reveal which patients are in fact active smokers and which are simply exposed to environmental tobacco smoke.

The advantage of this method is that it measures both compounds in a more rapid, reproducible and accurate manner than in previous HPLC studies [9, 12, 18, 19, 20].

The compounds (vitamin A and E and internal standards) are measured in the same sample at the same time.

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